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Cloning of a Methanol-Inducible *moxF* Promoter and Its Analysis in *moxB* Mutants of *Methylobacterium extorquens* AM1rif

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In *Methylobacterium extorquens* AM1, genes encoding methanol dehydrogenase polypeptides are transcriptionally regulated in response to C_1 compounds, including methanol (M. E. Lidstrom and D. I. Stirling, Annu. Rev. Microbiol. 44:27–57, 1990). In order to study this regulation, a transcriptional fusion has been constructed between a β-galactosidase reporter gene and a 1.55-kb *Xho*I-*Sal*I fragment of *M. extorquens* AM1rif DNA encoding the N terminus of the methanol dehydrogenase large subunit (*moxF*) and 1,289 bp of upstream DNA. The fusion exhibited orientation-specific promoter activity in *M. extorquens* AM1rif but was expressed constitutively when the transcriptional fusion was located on the plasmid. However, correct regulation was restored when the construction was inserted in the *M. extorquens* AM1rif chromosome. This DNA fragment was shown to contain both the *moxFJGI* promoter and the sequences necessary in *cis* for its transcriptional regulation by methanol. Transcription from this promoter was studied in the *M. extorquens* AM1rif *moxB* mutant strains UV4rif and UV25rif, which have a pleiotropic phenotype with regard to the components of methanol oxidation. In these mutants, β-galactosidase activity from the fusion was reduced to a level equal to that of the vector background when the fusion was present in both plasmid and chromosomal locations. Since both constitutive and methanol-inducible promoter activities were lost in the mutants, *moxB* appears to be required for transcription of the genes encoding the methanol dehydrogenase polypeptides.

Methylotrophic bacteria are capable of growth on reduced single-carbon compounds as the sole source of carbon and energy. The facultative methylotroph *Methylobacterium extorquens* AM1 can utilize one-carbon compounds, such as methanol and methylamine, as well as multicarbon compounds. Growth of *M. extorquens* AM1 on methanol requires the periplasmic enzyme methanol dehydrogenase (MDH) to oxidize the substrate to formaldehyde (4). Formaldehyde is then further oxidized to carbon dioxide or assimilated into cell carbon via the serine cycle (4).

A complex array of genes are involved in methanol oxidation (*mox*) in *M. extorquens* AM1 (16), and functions have been assigned to a number of them: *moxF* encodes the MDH large subunit (24), *moxI* encodes the MDH small subunit (23), *moxG* encodes the cytochrome c_L structural polypeptide (the electron acceptor of MDH) (22), and *moxA*, *moxK*, and *moxL* encode polypeptides apparently involved in the generation of active MDH from the pyrroloquinoline quinone prosthetic group and the apo-MDH (25). Other genes such as *moxB*, *moxE*, *moxM*, *moxN*, *moxD*, and *moxQ* have been postulated to play a role in regulation, stability, or processing owing to the pleiotropic phenotype of these mutants (16, 17, 25).

moxF, *moxG*, and *moxI* have been localized to an 8.6-kb *Hin*dIII DNA fragment from *M. extorquens* AM1 (HINDIII-FG) along with *moxJ*, encoding a 30-kDa polypeptide of unknown function (2). Expression studies (2) and DNA sequence analysis (3) have shown that these four genes are transcribed in the same direction in the order *moxFJGI*, and data from transposon Tn*5* insertion analyses (24) suggest that they may be cotranscribed. Recent data have suggested that a fifth gene (*moxR*) may be located immediately downstream of *moxI* (30). A single transcription start site has been mapped to 167 or 168 bp upstream of the *moxF* start codon (1, 3). It has also been shown that methanol-grown cells contain higher levels of MDH activity (20, 31), MDH polypeptides (31), and *moxF* mRNA (1, 3) than succinate-grown cells. Methylamine-grown cells also contain high MDH activity, and so the identity of the actual inducer is not known, but it appears to involve one or more C_1 compounds. Sequences upstream of *moxF* in *M. extorquens* AM1, *Methylobacterium organophilum* XX (18), *Paracoccus denitrificans* (13), and *Methylomonas albus* BG8 (16) and of *dcmA* in *Methylobacterium* sp. strain DM4 (15) do not reveal *Escherichia coli* σ^{70}-type consensus promoter sequences. Instead, a different sequence is present upstream of all of these genes, and a putative consensus promoter sequence based on sequence comparisons has been proposed (17). The putative promoter for *moxF* from *M. extorquens* AM1 lies 180 bp upstream of the start codon (3). However, nothing is known about the sequences involved in regulation by C_1 compounds.

We have initiated studies of *moxFJGI* transcription in *moxB* mutants. *moxB* mutants contain no immunodetectable MDH protein (large or small subunit) and no detectable cytochrome c_L (25). Therefore, *moxB*, located 11 kb downstream of *moxF* (24), is a candidate for a gene required for transcription of the *moxFJGI* cluster. In order to analyze the *moxB* function in more detail, a transcriptional fusion has been made between the region upstream of *moxF* and a *lacZ* reporter gene. Studies were undertaken in *M. extorquens* AM1rif, a Rif^r derivative of strain AM1, using plasmid and chromosomal constructs, to show that the fragment contains a promoter whose activity is increased during growth on methanol compared with that during growth on succinate. For convenience, we will refer to this as a methanol-inducible promoter. The activity of this promoter in *M. extorquens* AM1rif mutants UV4rif and UV25rif, both defective in the proposed regulation gene *moxB*, was investi-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant traits	Source or reference
E. coli		
DH5α	$r^- m^+$ *recA1 lacZYA* φ80d*lac*Δ(*lacZ*)M15	BRL, Inc.
MM294	*recA*$^+$	7
M. extorquens AM1 strains		
AM1rif	Rif^r derivative of AM1	24
UV4rif, UV25rif	*moxB* mutants of AM1rif	24
Plasmids		
410XSpro7	HINDIII-FG 1.55-kb *Xho*I-*Sal*I in pUC7, Ap^r	D. Anderson
pGD500	Promoterless *lacZY* transcriptional fusion vector, Tc^r	9
pGD323	*Xho*I-*Sal*I promoter in pGD500, incorrect orientation to *lacZ*, Tc^r	This study
pGD327	*Xho*I-*Sal*I promoter in pGD500, correct orientation to *lacZ*, Tc^r	This study
pTZ19TT	T7 promoter gene expression, multiple cloning site, translation terminators, Ap^r	2
pTZ1914	*lacZY* of pGD500 in *Sal*I site of pTZ19TT, Ap^r	This study
pBR322	Tc^r Ap^r	8
pCM301	pGD327 *moxF-lacZY* fusion in pBR322, Tc^r	This study
pRK2013	Mobilizing "helper" plasmid, Km^r	10

gated. The results confirmed the role of *moxB* in transcriptional regulation of the *moxF* region.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pGD500 was provided by G. Ditta, University of California, San Diego. D. Anderson constructed plasmid 410XSpro7 while at the University of Washington, Seattle.

Media and growth conditions. *M. extorquens* AM1 derivative strains were grown at 30°C on the ammonium-mineral salts medium described by Harder et al. (12) supplemented with a vitamin solution (29). Succinate was added to 0.2% (wt/vol), and methanol was added to 0.5% (vol/vol). For growth on methylamine, the medium was supplemented with both methylamine at 0.2% (wt/vol) and methanol at 0.2% (vol/vol). All *M. extorquens* AM1rif strains containing plasmids or with pCM301 inserted into the chromosome were always grown or maintained in the presence of tetracycline. *E. coli* strains were grown at 37°C in Luria broth (19). Agar was added to 1.5% (wt/vol) for plates. Antibiotics were added to sterile medium in the following concentrations: rifamycin, 20 μg/ml; tetracycline, 10 μg/ml; kanamycin, 50 μg/ml; and ampicillin, 100 μg/ml.

Bacterial matings. Triparental matings were performed as described previously (11). Mating mixtures were plated on succinate minimal medium when *M. extorquens* AM1rif strains were the recipients or on Luria agar when *E. coli* DH5α was the recipient.

DNA manipulations. Restriction enzyme digestions, ligations, plasmid isolations, and transformations of DNA into *E. coli* DH5α were carried out as described by Maniatis et al. (19). DNA sequencing was done by the dideoxy chain-termination method of Sanger et al. (28), with Sequenase and T7 sequencing primer (US Biochemical Corp., Cleveland, Ohio). Chromosomal DNA isolation from *M. extorquens* AM1rif strains was performed as described previously (27) with slight modifications: incubation with lysozyme at 37°C for 1 h before freezing at −70°C, and thawing in the presence of 1 mg of protease (pronase E; Sigma Chemical Co., St. Louis, Mo.) per ml at 56°C for 30 to 60 min, until cell lysis was complete.

DNA-DNA hybridizations. *Hin*dIII-digested chromosomal DNA samples were subjected to electrophoresis in Tris-acetate–0.8% (wt/vol) agarose gels (19). Gels were denatured, neutralized, dried, and then prehybridized at 42°C with 8× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% (wt/vol) sodium dodecyl sulfate (SDS)–1% (wt/vol) powdered milk. Hybridization of gels with DNA randomly prime labeled with [^{32}P]dCTP (Boehringer GmbH, Mannheim, Germany) was performed at 50°C with the same solution. Washes were done at 56°C with 0.5× SSC–0.1% (wt/vol) SDS.

Preparations of cell extracts. *M. extorquens* AM1rif strains were grown in liquid culture with appropriate additions of succinate, methanol, methylamine, vitamins, and antibiotics. Cells were harvested, washed once with the ammonium-mineral salts medium, recentrifuged, and resuspended in 2 ml of the same medium. The cells in the suspension were then broken by three passes through a French pressure cell at 20,000 lb/in^2. Cell suspensions were kept on ice, and the French pressure cell was chilled to 4°C. The cell extracts were centrifuged at 30,000 × *g* for 30 min, and the supernatants were decanted and stored at −20°C.

MDH assay. MDH activity was assayed by the phenazine methosulfate-dichlorophenol indophenol dye-linked method described previously (5, 31) with the following changes: 70 mM pyrophosphate buffer, pH 9.0; 10 mM NH_4Cl; 30 mM KCN; 1 mM phenazine methosulfate; 0.1 mM dichlorophenol indophenol; 70 mM methanol. An extinction coefficient of 22.5 μM for dichlorophenol indophenol was used in the calculations (6).

β-Galactosidase assay. β-Galactosidase activity was assayed as described by Miller (21).

Protein determination. Proteins were assayed with the Bio-Rad protein assay (Bio-Rad Lab., Richmond, Calif.). Stock solutions of bovine serum albumin were used as standards.

Western immunoblots. *M. extorquens* AM1rif cell extracts were separated by electrophoresis through 12.5% polyacrylamide–SDS gels. Polypeptides were then transferred to nitrocellulose (0.2 μm) as described by Maniatis et al. (19). MDH subunits were detected with anti-MDH primary antibody (2) and subsequent reaction with alkaline phosphatase-conjugated second antibody (Bio-Rad Lab.) (14).

RESULTS

Construction of *moxF-lacZY* transcriptional fusions. A 1.55-kb *Xho*I-*Sal*I fragment of *M. extorquens* AM1rif DNA, encoding 267 bp of *moxF* and 1,289 bp of upstream DNA (Fig. 1A), was used to determine whether the upstream DNA contained sequences that functioned in *M. extorquens* AM1rif as a methanol-inducible promoter. The fragment was linked to a promoterless β-galactosidase reporter gene by

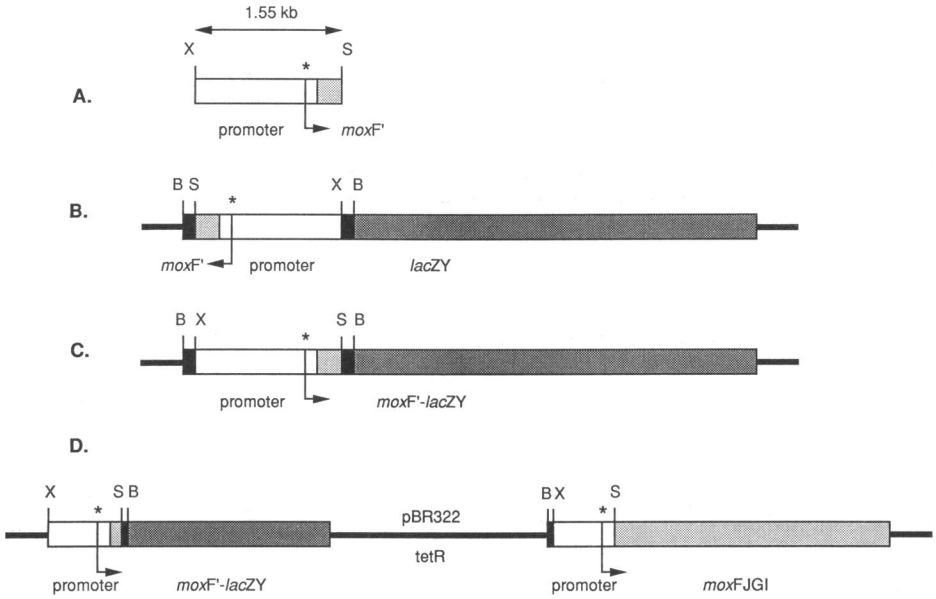

FIG. 1. Promoter fragment and transcriptional fusions constructed in this study. (A) The 1.55-kb *Xho*I-*Sal*I promoter fragment. It encodes 267 bp of the *moxF* structural gene (shaded) and 1,289 bp of upstream DNA. (B) Transcriptional fusion of the promoter fragment to *lacZY* of pGD500 in pGD323. Transcription reads away from the β-galactosidase gene. (C) Transcriptional fusion of the promoter fragment to *lacZY* of pGD500 in pGD327. Transcription reads into the β-galactosidase gene. (D) Transcriptional fusions that result from insertion of pCM301 into the chromosome. *lacZY* is transcribed from the chromosomal promoter, and *moxFJGI* is transcribed from the *Xho*I-*Sal*I fragment. The transcription start site at base 1121 from *Xho*I is noted (*). Restriction enzyme sites: X, *Xho*I; S, *Sal*I; B, *Bam*HI.

transcriptional fusion in pGD500, a low-copy-number plasmid with a broad host range (9).

Plasmid 410XSpro7 contains the *Xho*I-*Sal*I fragment cloned into the *Sal*I site of pUC7. The fragment was recovered by *Bam*HI digestion and cloned into the *Bam*HI site of pGD500. Two constructions were obtained, pGD323 (Fig. 1B) and pGD327 (Fig. 1C), which differ only in the orientation of the inserted fragment. pGD327 has the *Xho*I-*Sal*I fragment inserted so that transcription from the *moxF* start site reads into the β-galactosidase gene (correct orientation), whereas transcription from the *moxF* start site in pGD323 reads away from β-galactosidase (incorrect orientation).

Expression of β-galactosidase from *moxF*. Plasmids pGD500, pGD323, and pGD327 were mobilized into *M. extorquens* AM1rif by triparental matings. Transconjugants were isolated and grown in liquid culture with either succinate or methanol as the sole carbon source. The MDH and β-galactosidase activities determined from cell extracts of *M. extorquens* AM1rif with and without the plasmids are shown in Table 2. *M. extorquens* AM1rif cells containing no plasmid or containing pGD500, pGD323, or pGD327 had no detectable MDH activity when grown on succinate (noninducing conditions). In the same strains grown on methanol (inducing conditions), MDH activity increased to 0.09 to 0.2 μmol min^{-1} mg of $protein^{-1}$, with the lower levels being observed in cells containing plasmids with the *Xho*I-*Sal*I fragment. No β-galactosidase activity was observed in the absence of plasmids. Cells containing pGD500 had β-galactosidase activities of 0.14 to 0.16 μmol min^{-1} mg of $protein^{-1}$ under both growth conditions, which is the background level of noninducible β-galactosidase activity. Extracts of cells containing pGD323 had β-galactosidase activities similar to those of cells containing pGD500 for both growth substrates. However, extracts of cells containing pGD327 had approximately 100-fold-greater β-galactosidase activities. This indicated that the *Xho*I-*Sal*I fragment contained an orientation-specific promoter. However, the high β-galactosidase activity measured in extracts of cells containing pGD327 under noninducing conditions was not expected. It suggested that the promoter located on the plasmid was not regulated correctly with regard to growth on

TABLE 2. Enzyme activities from cell extracts

Strain	Activity[a] (μmol/min/mg of protein)			
	Methanol dehydrogenase		β-Galactosidase	
	Succinate	Methanol[b]	Succinate	Methanol[b]
AM1rif	0	0.22	0	0
AM1rif(pGD500)	0	0.20*	0.16	0.14
AM1rif(pGD323)	0	0.09*	0.12	0.12
AM1rif(pGD327)	0	0.10*	11.60	15.81
AM1rif::pCM301	0.01	0.16	0.82	4.97
UV4rif	0	0	0	0
UV4rif(pGD500)	0	0	0.12	0.07*
UV4rif(pGD323)	0	0	0.16*	0.10*
UV4rif(pGD327)	0	0	0.18	0.16*
UV4rif::pCM301	0	0	0.03	0.05
UV25rif	0	0	0	0
UV25rif(pGD500)	0	0	0.16*	0.10
UV25rif(pGD323)	0	0	0.16*	0.14*
UV25rif(pGD327)	0	0	0.12	0.13
UV25rif::pCM301	0	0	0.03	0.03

[a] All determinations were carried out at least twice, and values agreed within ±25%, except those indicated by *, which agreed within ±40%. 0, not detectable.

[b] For the UV4rif and UV25rif strains, methylamine was also present.

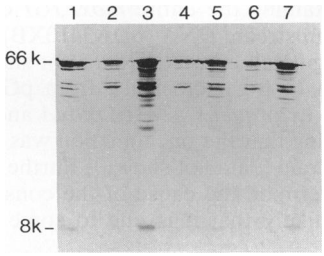

FIG. 2. Immunoblots of *M. extorquens* AM1rif cell extracts with antisera for the large (66-kDa) and small (8-kDa) MDH subunits. Lanes: 1, purified MDH (1 μg); 2, succinate-grown wild-type cells; 3, methanol-grown wild-type cells; 4, succinate-grown cells with pGD327; 5, methanol-grown cells with pGD327; 6, succinate-grown AM1rif::pCM301 cells; 7, methanol-grown AM1rif::pCM301 cells. Lanes 2 through 7 were each loaded with 25 μg of protein.

succinate. However, the low (nondetectable) level of MDH activity in succinate-grown cells containing pGD327 suggested that the chromosomal promoter was regulated normally.

Expression of MDH polypeptides in cells containing pGD327. MDH activity does not necessarily reflect production of MDH polypeptides, since other factors required for MDH activity, such as the pyrroloquinoline quinone cofactor, could be limiting under noninducing conditions. Therefore, to determine whether expression of MDH polypeptides was constitutive in cells containing pGD327, cell extracts were subjected to immunoblot analysis with anti-MDH antibody. Figure 2 shows extracts from succinate- and methanol-grown *M. extorquens* AM1rif cells containing either no plasmid (lanes 2 and 3) or pGD327 (lanes 4 and 5); purified MDH is also shown (lane 1). The bands between 66 and 20 kDa that cross-react with the MDH antiserum are degradation products of the 66-kDa (MoxF) subunit and are commonly observed in immunoblots (2, 25). Levels of the 66-kDa and 8-kDa (MoxI) polypeptides were higher in extracts of cells grown on methanol (Fig. 2, lanes 3 and 5) than in those of cells grown on succinate (lanes 2 and 4). These data indicate that the chromosomal promoter was not constitutively expressed in the presence of pGD327.

Construction of pCM301 for insertion into the chromosome. To determine whether incorrect regulation of the β-galactosidase transcriptional fusion of pGD327 under noninducing conditions could be eliminated, the fusion was introduced into the chromosome. Plasmid pCM301, which is incapable of replication in *M. extorquens* AM1rif and can only be maintained by insertion into the chromosome, was therefore constructed. The β-galactosidase gene was first cloned from pGD327 into pTZ19TT as a *Sal*I fragment, resulting in plasmid pTZ1914 (Table 1). The transcriptional fusion was reconstructed by insertion of the 1.55-kb *Bam*HI promoter fragment from pGD327 into the *Bam*HI site of pTZ1914. The correct orientation of the promoter fragment in relation to the β-galactosidase gene was confirmed by DNA sequencing from the T7 promoter. The transcriptional fusion was reclaimed by digestion with *Pst*I and cloned into the β-lactamase gene of pBR322 to form pCM301 (Table 1). pCM301 was mobilized into *M. extorquens* AM1rif, and transconjugants were selected for tetracycline resistance. Tetracycline-resistant transconjugants were generated by insertion of the entire plasmid into the chromosome through a single crossover event occurring at the homologous DNA sequences of the *moxF* promoter region. The final construction within the chromosome contained a transcriptional fusion of *lacZ* to the chromosomal *moxF* promoter and a second transcriptional fusion of the *moxFJGI* genes to the *Xho*I-*Sal*I promoter fragment. The two fusions are separated by pBR322 vector sequences (Fig. 1D). Insertion of pCM301 into the correct chromosomal location was confirmed by DNA-DNA hybridization analysis of chromosomal DNA isolated from *M. extorquens* AM1rif::pCM301 cells with labeled pBR322 and pCM301 as probes (data not shown).

Expression of β-galactosidase from *moxF* in the chromosome. Enzyme activities determined from cell extracts of *M. extorquens* AM1rif::pCM301 grown on succinate or methanol are shown in Table 2. The MDH activity was similar to that of the wild-type strain with no plasmid, being low under noninducing conditions and high under inducing conditions. A small amount of activity was detected in succinate-grown cells, but this was near the detection limit for the assay and may not be significant. Western blots confirmed that MDH polypeptides were induced in the presence of methanol (Fig. 2, lanes 6 and 7). These results indicated that the *Xho*I-*Sal*I promoter fragment, which was now fused to *moxFJGI* in the chromosome, contained a methanol-inducible promoter that was regulated normally. β-Galactosidase activity was also inducible by methanol in this strain, in which it was fused to the chromosomal *moxF* promoter, showing a sixfold increase under inducing conditions.

Expression of transcriptional fusions in *moxB* mutants. *M. extorquens* AM1rif strains UV4rif and UV25rif have lesions in *moxB* and are thought to be regulation mutants because of their pleiotropic phenotypes (16, 25). Plasmids pGD500, pGD323, pGD327, and pCM301 were mobilized into strains UV4rif and UV25rif to test the expression of the transcriptional fusion. The pCM301 insertions into *moxF* of UV4rif and UV25rif were confirmed by DNA-DNA hybridization (data not shown). Transconjugants were grown on succinate or methylamine plus methanol to provide noninducing and inducing conditions, respectively. Methylamine allows growth of the mutant, and methanol is provided to induce the *mox* genes. These growth conditions induce the MDH polypeptides in *moxA*, *moxK*, and *moxL* mutants (25) and should be sufficient for induction of *mox* genes in mutants unable to grow on methanol.

MDH and β-galactosidase activities were determined from cell extracts and are shown in Table 2. No MDH activity was detected in any extracts from *M. extorquens* strains UV4rif and UV25rif, and immunoblot analysis indicated that MDH polypeptides were not present (data not shown). β-Galactosidase activity was also not detected under either growth condition in cell extracts of strains UV4rif and UV25rif without plasmids. Extracts from cells containing plasmid pGD500 or pGD323 had β-galactosidase activities similar to those determined for wild-type cells containing the same plasmids under both growth conditions. Therefore, background β-galactosidase activity is the same in the wild-type and both mutant strains of *M. extorquens* AM1rif.

The β-galactosidase activities detected in extracts of *M. extorquens* strains of UV4rif and UV25rif containing pGD327 were markedly different from the activities in extracts of wild-type cells containing pGD327. Rather than the very high levels of β-galactosidase activity observed in wild-type cells, only low levels of β-galactosidase activity were detected in the mutant strains containing pGD327, regardless of the growth substrate. Cell extracts of *M. extorquens* strains UV4rif::pCM301 and UV25rif::pCM301 containing the chromosomal *moxFJGI* transcriptional fusions shown in Fig. 1D had β-galactosidase activity levels

below the background activity level measured for wild-type or mutant cells containing plasmids. These data indicate that no transcription of the *moxF* promoter occurred in the *moxB* mutant strains.

DISCUSSION

We have confirmed that the 1.55-kb *Xho*I-*Sal*I fragment of *M. extorquens* AM1rif DNA covering the 5′ end of *moxF* contains a promoter, as suggested from previous transcriptional start site mapping (1). The promoter was shown to be orientation specific by measuring β-galactosidase activities in extracts of cells containing plasmid pGD323 or pGD327. The direction of transcription from the *Xho*I-*Sal*I fragment agreed with that found by previous transcriptional analysis of *moxFJGI* (1). No promoter activity was detected in the direction opposite to the *moxF* promoter.

MDH activities, MDH polypeptides, and β-galactosidase activities measured in extracts of *M. extorquens* AM1rif::pCM301 cells demonstrated that the promoter contained within the *Xho*I-*Sal*I fragment was methanol inducible, that is, it had higher activity in methanol-grown cells than in succinate-grown cells. Previous studies have indicated that growth of *M. extorquens* AM1 on methanol usually results in an approximately sixfold induction of MDH activity (20, 31). However, MDH activities differ depending upon the growth conditions and substrate concentration (20, 31). A sixfold increase in β-galactosidase activity was observed when *lacZ* was transcribed from the normal chromosomal promoter in AM1rif::pCM301 cells, and the immunoblots suggest an increase in MoxF and MoxI polypeptides in the same range. Under the growth conditions described here, succinate-grown cells usually did not show sufficient MDH activity for detection (Table 2). This presumably reflects other limiting factors for MDH activity rather than the level of the structural polypeptides and suggests differential regulation of methanol oxidation functions. Previous data have shown that the levels of *moxF* transcripts are 5- to 10-fold higher in methanol-grown *M. extorquens* AM1 than in succinate-grown cells (1, 3). However, it was not known whether this regulation was due to changes in transcription or in mRNA stability. Our data showing that β-galactosidase activities in extracts of *M. extorquens* AM1rif::pCM301 cells were sixfold higher in methanol-grown cells than in succinate-grown cells indicate that part or all of this regulation is due to changes in transcription.

The β-galactosidase activities measured in *M. extorquens* AM1rif cells containing pGD327 suggested that transcription from the plasmid-borne *Xho*I-*Sal*I fragment was constitutive when cells were grown under inducing and noninducing conditions. Although the reason for this incorrect regulation is not known, a few possibilities have already been eliminated in this study. An obvious explanation would be titration of a repressor molecule by an increase in the number of binding sites. If that were true, MDH polypeptide levels and possibly MDH activities would have been expected to increase dramatically under noninducing conditions in all cells carrying plasmids containing a *moxF* promoter. In fact, MDH activity was lower, as much as half of the level in cells without extra binding sites, and MDH polypeptides showed only a small increase (Table 2 and Fig. 2). Another possible reason for constitutive expression of β-galactosidase could be the plasmid location if a *cis*-acting effect, such as DNA looping (26), was required for normal regulation. However, MDH activities and polypeptides in extracts of cells containing a similar plasmid without a *lacZ* fusion but containing the entire *moxFJGI* cluster with exactly the same upstream DNA (pDA3410XB) (2) were determined to be regulated correctly (data not shown). Constitutive expression of β-galactosidase from pGD327 was also observed in *M. extorquens* AM1rif *moxA* and *moxK* mutant strains, indicating that the phenomenon was not confined to the wild-type strain (data not shown). Further studies will be required to determine the cause of the constitutive expression, but it is likely that it is due to some artifact of this construction.

Regardless of the underlying mechanism, the incorrect regulation of the *moxF-lacZ* transcriptional fusion in pGD327 suggests that this construction is inappropriate for further analysis of *moxF* promoter regulation. However, insertion of the same construct into the chromosome has provided an alternative system in which regulation of *lacZ* and *moxFJGI* can be studied and compared, as single copies, each under the control of a *moxF* promoter.

These studies were initiated with an investigation of promoter activity in the putative regulatory mutant strains *M. extorquens* UV4rif and UV25rif, with lesions in *moxB*. No MDH activity was observed in extracts from these cells, as MDH polypeptides are not present (25). The β-galactosidase activities measured in extracts from mutant cells containing pGD500, pGD323, or pGD327 were all similar to background β-galactosidase activities determined in extracts of wild-type cells containing plasmid pGD500 or pGD323. Extracts of *M. extorquens* UV4rif::pCM301 and UV25rif::pCM301 cells had further reduced β-galactosidase activity, probably due to the single copy of *lacZ* rather than the multiple copies in the cells carrying plasmids. These data indicate that no significant transcription from the *moxF* promoter occurs in the mutant strains UV4rif and UV25rif, regardless of whether it is located on a plasmid or in the chromosome. *moxB* must therefore encode a polypeptide essential for transcription from the *moxF* promoter, either a transcription factor or a function involved in generating an active transcription factor. This transcription factor could be an activator protein or a sigma factor.

Further work will be required to determine the function of *moxB*, including DNA sequence analysis and in vitro transcription studies. However, the work presented here clearly shows that *moxB* is essential for transcription of the *moxFJGI* region in *M. extorquens* AM1rif.

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